Functional non-identity of creatine kinase subunits of rabbit skeletal muscle

G.A. Nevinsky, V.N. Ankilova, O.I. Lavrik, Z.S. Mkrtchyan*, L.S. Nersesova* and J.I. Akopyan

Institute of Organic Chemistry, Novosibirsk 630090 and **Institute of Experimental Biology, Yerevan, USSR*

Received 6 September 1982

Rabbit skeletal muscle *Creatine kinase subunit* *Transphosphorylation* *Subunit function* *Active centre identity* *Functional non-identity*

1. INTRODUCTION

Creatine kinase catalyzes the reversible transfer of a phosphoryl group from $MgATP^{2-}$ to creatine. The enzyme from rabbit skeletal muscle consists of 2 very similar, if not identical subunits of M_r 41 000 [1]. From the functional behaviour of CK identity of the enzyme subunits were proposed [1,2]. However, using a fluorescent probe technique, it was reported [3] that the binding of ADP to CK in the presence of creatine and nitrate displayed features similar to negative cooperativity. We have shown [4,5] that γ-arylamides of ATP and ϵATP exhibit two K_d-values, which differ 3–10-fold. The non-identical behaviour could arise from non-identity of active sites on different subunits of CK. The subunits might be identical, but dimer exhibits two K_d-values due to asymmetric association of the subunits or conformational changes induced by ligand binding. Here an attempt is made to distinguish between these alternatives. We have effected the separation of the different subunits of CK by affinity chromatography on Sepharose containing an immobilized analog of ATP, after dissociation of CK into subunits by 5 M urea, and have shown their functional non-identity.

Abbreviations: CK, creatine kinase (EC 2.7.3.2); azido-ATP, γ-(*p*-azidoanilide)-ATP; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TNS, 6-(4-toluidino)-2-naphthalenesulfonic acid

2. MATERIALS AND METHODS

Phosphocreatine, ADP and ATP were from 'Reanal' (Hungary), creatine was from 'Chemapol' (Czechoslovakia), Hepes was from 'Ferak' (FRG). All other reagents were of analytical grade. CK from rabbit skeletal muscle was purified to homogeneity according to [6]. The enzyme concentration was determined spectrophotometrically, using E_{280} 7.4×10^4 $cm^{-1}.M^{-1}$ [7]; CK spec. act. was 200–400 U/mg.

The synthesis and characterization of azido-ATP were described in [8].

The measurements of the enzyme actvity in the reaction of phosphocreatine synthesis (forward reaction) were performed pH-metrically at 30°C according to [9]. The reaction mixture (1.5 ml) was 40 mM in creatine, 4 mM in ATP, 5 mM in $MgAc_2$, 1 mM in β-mercaptoethanol, 0.1 mM in EDTA. In experiments at pH 8.5–9.5 composition of the reaction mixture was the same except for being 0.1 M in NaAc.

CK activity in the reaction of transfer of phosphoryl group from phosphocreatine to ADP (reverse reaction) was tested by colourimetrical method [10] at 30°C according to [11]. The reaction mixture (0.15 ml) was 1–5 mM in ADP, 5–15 mM in phosphocreatine, contained $MgAc_2$ in 3-fold molar excess over ADP and was 0.1 M in buffer; at pH 8–10 the buffer was Hepes–KOH; pH 6.5–8.0, Tris–acetate buffer; pH 5–6, NaAc-buffer. The reaction was allowed to proceed by ad-

Published by Elsevier Biomedical Press

001457593/82/0000—0000/$2.75

dition of phosphocreatine. After 1–2 min the reaction was stopped by addition of 0.2 ml alkaline mixture (16 g Na_2CO_3, 6 g NaOH, H_2O to 100 ml) containing 1% α-naphtol; 0.1 ml 0.1% diacetyl solution and 2 ml water. The resulting A_{520} of the solution was measured.

For preparation of isolated subunits, CK was dissolved (0.01–0.4 mg/ml) in buffer 1:5 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and applied to a column of Sepharose 4B containing immobilized (6-aminohexyl)-γ-amide of ATP (1.3 μmol ATP analog/ml resin). The first protein peak was eluted by buffer 1, the second one contained only M′-subunit of the enzyme and was eluted by buffer 1 plus 0.5–1.0 M NaCl. Complete separation of M- and M′-subunits was achieved by similar chromatography after incubation of CK (0.2–0.5 mg/ml, 30°C, 1 h) in buffer 1 containing 5–7 M urea.

Photo-induced modification of the enzyme was performed according to [9, 11] by UV-irradiation of mixture of CK with azido-ATP at 10°C using an SVD-120A mercury lamp and filter transmitting light of wavelength >300 nm.

Polyacrylamide disc electrophoresis was done in 7.5% gel using system with Tris–glycine buffer (pH 8.3) and 0.1% SDS according to [12]. Gels were stained with Coomassie brilliant blue R-250. The protein bands were detected with a densitometer (SCAN-400, Joyce-Loebl, England).

3. RESULTS AND DISCUSSION

CK can be readily dissociated into active subunits by urea or guanidinium chloride. Removal of the denaturating agents leads to reassociation of the subunits and restoration of the initial activity. Electrostatic interactions bring a significant contribution into the interaction CK with ADP or ATP. The addition of urea to the solutions does not destroy the electrostatic interactions [13]. Assuming the initial non-identity of CK subunits, we attempted to separate different subunits by chromatography on Sepharose containing an immobilized analog of ATP after dimer dissociation into subunits by 5 M urea. The result of the chromatography is shown in fig. 1. Of the protein material (A_{280}) 50% was eluted from the resin by buffer 1 and the rest by buffer 1 with 0.5–1.0 M NaCl. When a freshly prepared solution of lyophilized CK (0.1–0.4 mg/ml) was applied to the column protein retention did not exceed 5–7% (fig. 1), but after incubation of the enzyme solution (20°C, 1–3 h) the retention increased up to 20%. Application of a diluted solution of CK (1–10 μg/ml) gave up to 30% protein retention.

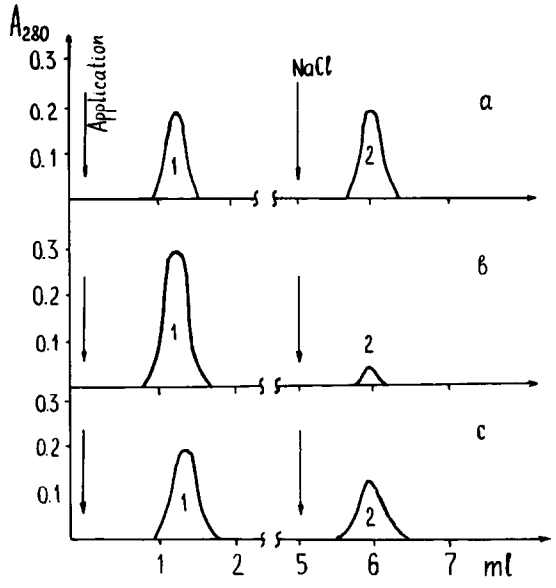

Fig. 1. Affinity separation of CK M- and M′-subunits on ATP–Sepharose. Peak 1 corresponds to M-subunit of CK; 2-M′-subunit. The subunit separation: (a) after incubation of CK (0.2 mg/ml, 1 h, 30°C) in buffer 1 plus 5 M urea; (b) freshly prepared solution of lyophilized CK (0.2 mg/ml) in buffer 1 without urea; (c) after incubation of CK (10 μg/ml, 1 h, 20°C) in buffer 1.

The enzyme activity and electrophoretic mobility of the protein materials of peak 1 and 2 after removal of urea and NaCl were tested. Both peak 1 (fig. 2a) and peak 2 (fig. 2b) exhibited only one protein band when tested by electrophoresis in the presence of 0.1% SDS. The mixture of aliquots of peak 1 and peak 2 exhibited two closely disposed bands as in the case of the untreated CK. Comparison of the intensities of protein bands (fig. 2a–c) shows that peak 1 corresponds to the less mobile M-subunit of CK and peak 2 to the more mobile M′-subunit. The difference in the mobility of M- and M′-components is negligible but reliable and is in agreement with results of [14].

The pH-dependence of the enzyme activity of

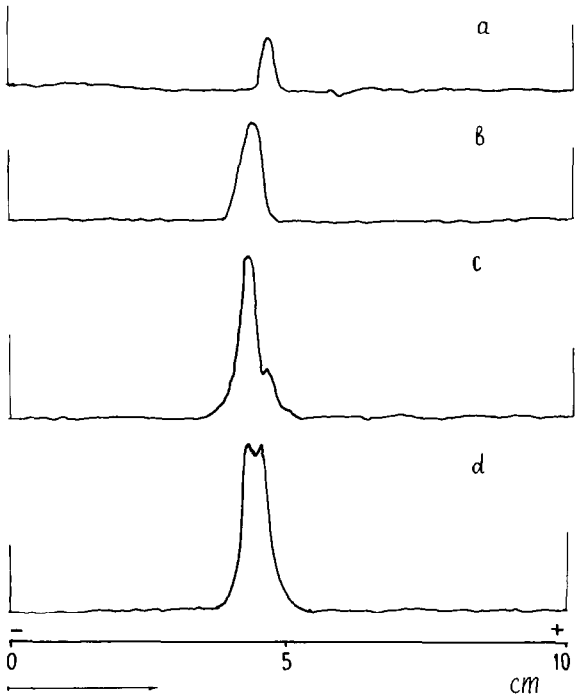

Fig. 2. Densitograms of gels after SDS–polyacrylamide electrophoresis of CK preparations. CK consisting only of: (a) M′-subunits (peak 2, fig. 1); (b) M-subunits (peak 1, fig. 1); (c) a mixture of aliquots of peaks 1 and 2 (see fig. 1); (d) untreated preparation of CK; 8–20 μg protein was applied/gel.

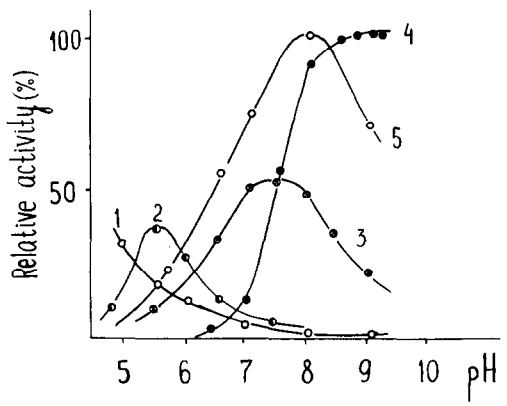

Fig. 3. pH-activity curves for different preparations of CK. Curves: (1, 5) activity measured in the reverse direction for CK preparations consisting only of M′- and only of M-subunits, respectively; (2, 4) in the forward reaction for CK preparations consisting only of M′- and only M-subunits, respectively; (3) for untreated CK in the reverse reaction; 100% of the activity corresponds to the activity of MM-kinase.

the MM and M′M′ forms of CK in both direct and reverse reactions are presented in fig. 3. MM-kinase catalyzed both reactions but at different pH-optima: 8, 5–9.5 and 7.7–8.5, respectively. M′M′-enzyme catalyzed direct and reverse reactions also. The pH-optima for these reactions corresponds to pH 5.3–5.8 and <5.0. The specific activity of MM-kinase was ~3–4-times higher than that of M′M′-enzyme. The preparations of MM-kinase were more stable during isolation and following storage.

The specific activity of CK preparations depends essentially upon the enzyme concentration in the reaction mixture (fig. 4). For instance, the M-component specific activity (a) of both MM-kinase and untreated MM′-enzyme decreases with the increasing protein concentration $[E]_o$. The data in fig. 4 allow us to suggest formation of less active dimeric MM- and MM′-forms of CK at high protein concentrations. The presence of CK monomeric form

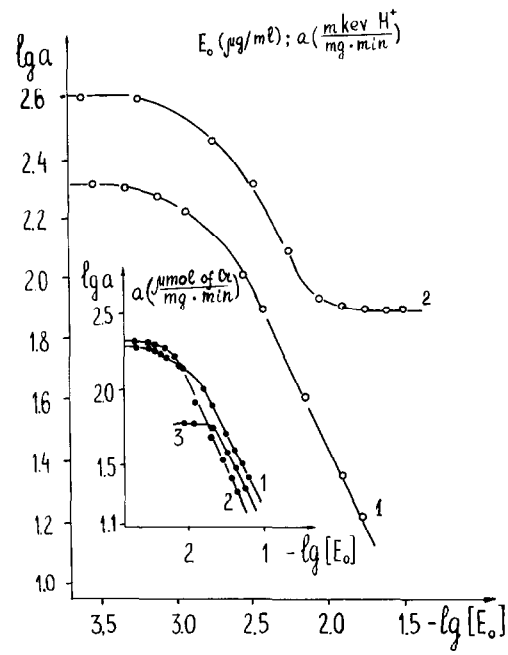

Fig. 4. The dependence of CK specific activity on [protein]. Curve: (1) corresponds to an untreated preparation of CK; (2) a preparation of CK consisting only of M-subunits. The activity was measured in the forward reaction at 30°C (pH 9.0). Insert: dependence of specific activity of untreated CK in the reverse reaction on [protein] at different pH-values. Curve: (1) pH 8.5; (2) pH 7.2; (3) pH 5.3.

in the solutions of the enzyme at pH 6–9 has been shown by a gel-filtration method [15].

At sufficiently low concentration of CK in the reaction mixture the dependence of log a on log $[E]_o$ for MM-kinase reaches a plateau corresponding to the specific activity (a_1) of the monomeric form of the enzyme and at high protein concentrations the dependence reaches a plateau corresponding to the specific activity (a_2) of MM-dimer. The different preparations of MM-kinase exhibited different values of the activity (a_1 and a_2) but in all cases the ratio of a_1/a_2 was equal to 5. The ratio of a_1/a_2 for untreated MM′-kinase as seen from (fig. 4) is >10. Such a decrease of the specific activity of CK on association of the subunits into MM- and MM′-dimers may be due to steric screening of the active sites during dimers formation, as is the case for other enzymes [16].

From the dependence of the specific activity of MM′-kinase upon the enzyme concentration in the reverse reaction at pH 5.3 and 8.5 it follows that the activity of M-subunit is 2–3-times higher than that of M′-subunit (fig. 4).

An additional confirmation of the non-identical behaviour of subunits came from investigation of the kinetics of enzyme modification by azido-ATP. Azido-ATP is an affinity reagent for CK [9]. UV-irradiation of the enzyme (pH 8.0) in the presence of the analog leads to an irreversible inactivation of the enzyme. ADP protects CK against inactivation (fig. 5). The enzyme activity of the M-subunit

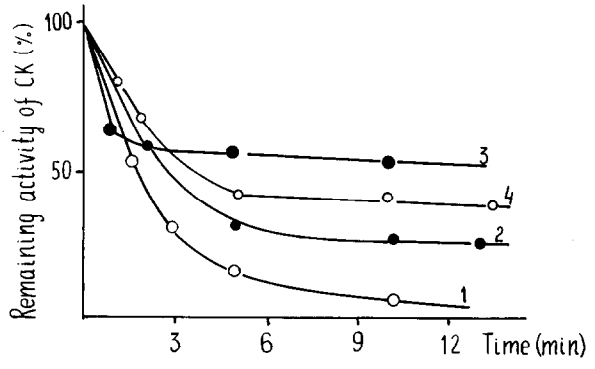

Fig. 5. Time dependence of the activity of untreated CK irradiated in the presence of azido-ATP (43 μM, pH 8.0). The remaining activity was measured in the reverse reaction at pH 8.5 (curves 1, 3) and pH 5.3 (curves 2, 4). In the case of curves 3, 4, ADP (5 mM) was added to irradiation mixture.

decreased more rapidly than the activity at pH 5.0 which corresponds predominantly to the activity of the M′-subunit. ADP protects only the M-subunit whereas protection of M′-subunit is negligible. In the absence of the other ligands the covalent binding of azido-ATP corresponds to 1.8 mol analog/mol dimer. ADP (1 mM) protects the enzyme against covalent binding of 1.1–1.2 mol analog/mol enzyme. The protection of the enzyme is incomplete even at a higher concentration of ADP (10 mM). The incomplete protection of the enzyme activity and lack of protection against covalent binding of 0.6–0.7 mol analog/mol enzyme under conditions used may be the result of modification of the M′-subunit. This may be due to formation of a ternary complex: CK · azido-ATP · ADP as with the affinity reagents ϵ-ATP γ-(*p*-azidoanilide) [5,17] and ϵ-ADP β-(*p*-azidoanilide) [11]. The formation of such a complex appears to be the result of interaction of the aromatic group on the terminal phosphate with the region of CK near the active center [11,17]. Covalent binding of the analog's reactive group with its acceptor on the enzyme does not distort the nucleotide-binding centers of the enzyme [11].

The different velocities of modification of M- and M′-subunits in the cases of ϵ-ATP γ-(*p*-azidoanilide) and ϵ-ADP β-(*p*-azidoanilide) were also obtained [5,11].

The non-identical behaviour could arise from asymmetrical association of subunits into dimer or asymmetry induced by nucleotides. The different behaviour of the subunits under affinity chromatography, different electrophoretic mobility of the subunits, different stability of the subunits and different pH-dependence of the activity of the M- and M′-components of the enzyme may indicate that M- and M′-subunits have non-identical active centers.

Three isozymes of CK from human serum: MM, MM′, M′M′ were revealed by isoelectric focusing and hybridization [18]. We have found 2 protein bands for MM-type CK from human cardiac muscle after SDS–polyacrylamide gel electrophoresis. A fluorescent probe technique in the presence of TNS has revealed two K_d-values for ADP, ATP and aminoalkylamides with 5–10-fold difference.

The results obtained allow us to suggest that dimeric structure of CK is not essential for expression of catalytic activity. The enzyme consists of

functionally different subunits. The subunits appear to have non-identical active centers. Functional non-identity of the subunits may reflect various catalytic and regulatory aspects of the transphosphorylation process.

ACKNOWLEDGEMENT

It is a pleasure to express our thanks to Professor D.G. Knorre for his interest to this work.

REFERENCES

[1] Bickerstaff, G.F. and Price, N.C. (1978) Int. J. Biochem. 9, 1–8.
[2] Watts, D.C. (1973) in: The Enzymes (Boyer, P.D. ed) vol. 8, pp. 383–455, Academic Press, London, New York.
[3] McLaughlin, A.C. (1974) J. Biol. Chem. 249, 1445–1452.
[4] Gorshkova, I.I., Lavrik, O.I. and Popov, R.A. (1981) Biokhimiya 46, 1564–1569.
[5] Denisov, A. Yu., Nevinsky, G.A. and Lavrik, O.I. (1982) Biokhimiya 47, 184–190.
[6] Chetverikova, E.P., Krinskaya, A.V., Rosanova, N.A., Alievskaya, L.L. and Rybina, V.V. (1970) Biokhimiya 35, 953–961.
[7] Milner-White, E.J. and Watts, D.S. (1971) Biochem. J. 122, 721–740.
[8] Nevinsky, G.A., Lavrik, O.I., Favorova, O.O. and Kisselev, L.L. (1979) Bioorg. Khim. 5, 352–364.
[9] Akopyan, J.I., Gazaryants, M.G., Mkrtchyan, Z.S., Nersesova, L.S., Lavrik, O.I. and Popov, R.A. (1981) Biokhimiya 3, 262–269.
[10] Ennor, A.H. and Rosenberg, H. (1952) Biochem. J. 51, 606–610.
[11] Denisov, A. Yu., Nevinsky, G.A. and Lavrik, O.I. (1982) Bioorg. Khim. 8, 72–79.
[12] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[13] Tener, G. (1970) in: Methods of Nucleic Acid Investigation (in Russian) (Belozersky, A.N. ed) pp. 85–90, Myr, Moscov.
[14] Williamson, H., Gree, K., Cherif, S. and Milner-White, E.J. (1977) Biochem. J. 167, 731–737.
[15] Rozanova, N.A. and Chetverikova, E.P. (1981) Biokhimiya 46, 2125–2135.
[16] Kurganov, B.I. (1978) in: Allosteric enzymes (in Russian), pp. 117–174, Nauka, Moscow.
[17] Nevinsky, G.A. and Denisov, A. Yu. (1981) Bioorg. Khim. 7, 1693–1700.
[18] Wevers, R.A., Wolters, R.J. and Soons, J.B.Y. (1977) Clin. Chim. Acta 78, 271–276.